

Role of Ion Channels and Exchangers in Mechanical Stretch-Induced Cardiomyocyte Hypertrophy

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Abstract—We have previously reported that stretching of cardiomyocytes activates the phosphorylation cascade of protein kinases, including Raf-1 kinase and mitogen-activated protein (MAP) kinases, followed by an increase in protein synthesis partly through enhanced secretion of angiotensin II and endothelin-1. Membrane proteins, such as ion channels and exchangers, have been postulated to first receive extracellular stimuli and evoke intracellular signals. The present study was performed to determine whether mechanosensitive ion channels and exchangers are involved in stretch-induced hypertrophic responses. Neonatal rat cardiomyocytes cultured on expandable silicone dishes were stretched after pretreatment with a specific inhibitor of stretch-sensitive cation channels (gadolinium and streptomycin), of ATP-sensitive K^+ channels (glibenclamide), of hyperpolarization-activated inward channels (CsCl), or of the Na^+-H^+ exchanger (HOE 694). Pretreatment with gadolinium, streptomycin, glibenclamide, and CsCl did not show any inhibitory effects on MAP kinase activation by mechanical stretch. HOE 694, however, markedly attenuated stretch-induced activation of Raf-1 kinase and MAP kinases by $\approx 50\%$ and 60% , respectively, and attenuated stretch-induced increase in phenylalanine incorporation into proteins. In contrast, HOE 694 did not inhibit angiotensin II- and endothelin-1-induced Raf-1 kinase and MAP kinase activation. These results suggest that among many mechanosensitive ion channels and exchangers, the Na^+-H^+ exchanger plays a critical role in mechanical stress-induced cardiomyocyte hypertrophy. (*Circ Res.* 1998;82:430–437.)

Key Words: mechanosensitive ion channel ■ Na^+-H^+ exchanger ■ Raf-1 kinase ■ mitogen-activated protein kinase

Mechanical stress activates a variety of protein kinases, such as Raf-1 and MAP kinases, in myocardial cells and induces cardiomyocyte hypertrophy.^{1,2} These hypertrophic responses are induced partly through an increase in secretion and synthesis of vasoactive peptides, such as Ang II and ET-1.^{3–6} However, it still remains to be determined how mechanical stress is converted into biochemical signals that activate protein kinase cascades and stimulate secretion and synthesis of the peptides.

Ion channels and exchangers in the cell membrane rapidly respond to extracellular stimuli⁷ and may be good candidates for “mechanoreceptors.” Mechanosensitive channels of various ionic selectivities have been found in many types of cells, including cardiac myocytes.^{8–15} Stretch-sensitive cation channels nonselective for Na^+ and K^+ exist in neonatal rat ventricular myocytes,⁸ and gadolinium and streptomycin block these channels.^{9,10} These channels have been implicated to mediate stretch-induced arrhythmias.¹¹ ATP-sensitive K^+ channels have also been identified in several different tissues, including cardiac myocytes.¹² Recently, it has been reported that opening these channels shows a marked cardioprotective effect in stunned myocardium and that an antagonist of these channels

(glibenclamide) worsens contractile function after reperfusion.¹³ Pacemaker inward channels, which are responsible for diastolic depolarization, can be blocked by CsCl.^{14,15} All of these channels have been demonstrated to be activated by mechanical stretch.⁷

The NHE, an electroneutral cotransport system, is a membrane glycoprotein present in most eukaryotic cells.¹⁶ The NHE regulates pH_i by controlling transepithelial transport of Na^+ and H^+ , ie, Na^+ influx and H^+ efflux with a stoichiometry of one to one. The NHE can be activated by a wide variety of mitogenic stimuli, including mechanical stretch,^{17–19} and its activation results in cytoplasmic alkalization, which may induce proliferation in many cell types.¹⁷ In addition, the activity of the NHE is increased in platelets,²⁰ lymphocytes,²¹ and red blood cells^{22,23} of patients with essential hypertension and in vascular smooth muscle cells²⁴ and kidney proximal tubules²⁵ of spontaneously hypertensive rats.

Therefore, we examined the involvement of these mechanosensitive ion channels and exchangers in the activation of protein phosphorylation cascade followed by increased protein synthesis in cultured cardiac myocytes. Although all inhibitors of mechanosensitive ion channels did not affect stretch-

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Selected Abbreviations and Acronyms

Ang II	= angiotensin II
ET-1	= endothelin-1
FAK	= focal adhesion kinase
HOE 694	= 3-methylsulfonyl-4-piperidinobenzoyl guanidine
MAP	= mitogen-activated protein
MBP	= myelin basic protein
MEK1	= (MAP kinase/extracellular signal-regulated kinase) kinase
NHE	= Na ⁺ -H ⁺ exchanger
Raf-1	= Raf-1 kinase
rMAPKK	= recombinant MAP kinase kinase

induced hypertrophic responses, a specific inhibitor of the NHE, HOE 694,²⁶ markedly inhibited mechanical stretch-induced activation of Raf-1 and MAP kinases and an increase in amino acid uptake into proteins. In contrast, activation of Raf-1 by Ang II and ET-1 was not affected by HOE 694. These results suggest that mechanosensitive ion channels examined in the present study are not involved in the mechanical stress-evoked hypertrophic responses in cardiac myocytes, whereas the NHE plays a critical role in converting mechanical stress into biochemical signals. Moreover, autocrinely released Ang II or ET-1 may not be related to mechanical stress-induced NHE activation.

Materials and Methods

HOE 694,²⁶ BQ 123, and CV 11974 were gifts from Hoechst Japan Ltd (Tokyo, Japan), Banyu Pharmaceutical Co Ltd (Tokyo, Japan), and Takeda Chemical Industries, Ltd (Osaka, Japan), respectively. [γ -³²P] ATP and [³H]phenylalanine were purchased from Du Pont-NEN Co; syntide-2 and PD 98059, from Santa Cruz Biochemistry, Inc. Other reagents were purchased from Sigma Chemical Co.

Cell Culture and Stretching of Cardiomyocytes

Primary cultures of cardiomyocytes were prepared from ventricles of 1-day-old Wistar rats, as described previously,²⁷ according to the method of Simpson and Savion²⁸ with minor changes. Stretching of myocytes was conducted as described previously.^{27,29} Beating cardiomyocytes cultured on expandable silicone rubber dishes were stationarily stretched by 20% and lysed on ice with the lysis buffer (25 mmol/L Tris-HCl, 25 mmol/L NaCl, 1 mmol/L sodium orthovanadate, 10 mmol/L NaF, 10 mmol/L sodium pyrophosphate, 10 mmol/L okadaic acid, and 0.5 mmol/L EGTA). Stretch and control experiments were performed simultaneously with the same pool of cells in each experiment to match for temperature, CO₂ content, or pH of the medium between stretched and control cells. It has previously been reported that as the length of the dish increases, the length of the cardiomyocyte parallel to the axis of deformation also increases to the same extent.^{27,30}

MAP Kinase Assay in MBP-Containing Gels

MAP kinase activities were measured using MBP-containing gels as described previously.¹ In brief, MAP kinases were immunoprecipitated with a polyclonal antibody against 42- and 44-kD MAP kinases and were electrophoresed on an SDS-polyacrylamide gel containing MBP. MAP kinases in the gel were denatured by guanidine/HCl and renatured in Tris-HCl solution. After incubation with [γ -³²P]ATP, the gel was washed extensively, dried, and subjected to autoradiography. The activities were determined by two-dimensional densitometer analysis of the autoradiographic intensity.

Assay of MAP Kinase Kinase Kinase Activity of Raf-1

Raf-1 has been reported to have MAP kinase kinase kinase activity.^{31,32} To analyze MAP kinase kinase kinase activity of Raf-1, we performed

two kinds of methods. First, the activities were assayed by measuring the phosphorylation of recombinant MAP kinase kinase fused to glutathione S-transferase (rMAPKK)³³ as described previously.² Immunoprecipitates obtained with an anti-Raf-1 antibody were incubated with rMAPKK and [γ -³²P]ATP. After incubation, rMAPKK was collected using glutathione beads and electrophoresed on a polyacrylamide gel. The gel was dried and subjected to autoradiography. Values are the mean of duplicate densitometric determinations from four experiments. Second, the activities were assayed by measuring the phosphorylation of syntide-2, a peptide substrate for Raf-1, as described previously.⁶ The lysates of myocytes were incubated with syntide-2 and [γ -³²P]ATP. After incubation, syntide-2 was collected using a P81 paper, which was washed in phosphoric acid and in acetone, dried, and counted by the Cerenkov method.

Amino Acid Uptake Into Cardiomyocytes

After being cultured in the serum-free medium for 48 hours, cardiomyocytes were stretched by 20% for 24 hours. The relative amount of protein synthesis was determined by assessing the incorporation of the radioactivity into cells as described previously.² [³H]Phenylalanine (1 μ Ci/mL) was added to the culture medium 2 hours before harvest. The cells were rapidly rinsed with ice-cold phosphate-buffered saline (10 mmol/L sodium phosphate and 0.85% NaCl, pH 7.4) and incubated for over 20 minutes on ice with trichloroacetic acid. The total radioactivity in each dish was determined by liquid scintillation counting.

Statistics

Differences within groups were compared by one-way ANOVA and Dunnett's *t* test. The accepted level of significance was *P* < .05.

Results

Involvement of Ion Channels and the NHE in Stretch-Induced MAP Kinase Activation

We first examined whether mechanosensitive ion channels are involved in stretch-induced activation of MAP kinases. After pretreatment with specific inhibitors of stretch-sensitive cation channels (gadolinium, 10⁻⁵ mol/L; streptomycin, 5 × 10⁻⁴ mol/L), of ATP-sensitive K⁺ channels (glibenclamide, 10⁻⁶ mol/L), or of hyperpolarization-activated inward channels (CsCl, 2 × 10⁻⁵ mol/L), cultured cardiomyocytes of neonatal rats were stretched by 20% for 8 minutes. Each agent has been reported to completely block each ion channel at the above-mentioned concentration.^{9,10,34-37} Mechanical stretch rapidly increased the activity of both 42- and 44-kD MAP kinases by \approx 3- to 4-fold, as reported previously.¹ Stretch-induced MAP kinase activation was not affected by pretreatment with any inhibitor of stretch-sensitive cation channels, such as gadolinium, streptomycin, glibenclamide, or CsCl (Fig 1). In contrast, when cardiac myocytes were pretreated with an NHE inhibitor, HOE 694 (10⁻⁵ mol/L), stretch-induced MAP kinase activation was attenuated by \approx 60% (Fig 1). The inhibitory effect on stretch-induced MAP kinase activation was observed from 10⁻⁸ mol/L HOE 694 and was concentration dependent (Fig 2). The activation was maximally inhibited by 10⁻⁶ mol/L HOE 694, but \approx 40% of the increased activity remained. Although the inhibitory effect of 10⁻⁵ mol/L HOE 694 appears to be smaller than that of 10⁻⁶ mol/L HOE 694, there was no significant difference between two groups (*P* = .60). These results suggest that MAP kinase activation evoked by mechanical stretch is partly dependent on the activated NHE.

HOE 694 Partly Blocks Stretch-Induced Activation of Raf-1

To determine whether the effects of HOE 694 are specific to activation of MAP kinases, we investigated another kinase, Raf-1,

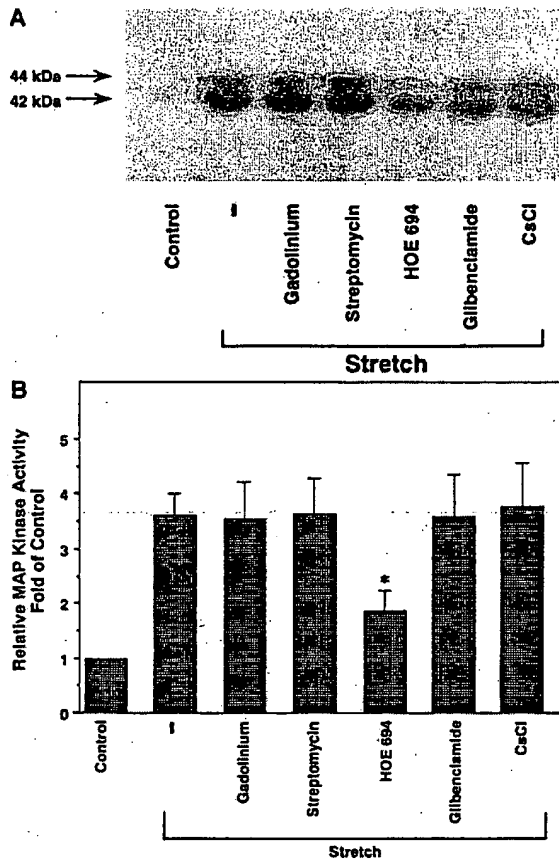


Figure 1. Effects of ion channel blockers and the NHE inhibitor on stretch-induced MAP kinase activation. After pretreatment with 10^{-5} mol/L gadolinium or 5×10^{-4} mol/L streptomycin for 60 minutes, 10^{-5} mol/L HOE 694 for 30 minutes, 10^{-6} mol/L glibenclamide for 60 minutes, or 2×10^{-3} mol/L CsCl for 30 minutes, cardiomyocytes were stretched by 20% for 8 minutes. MAP kinase activities were measured as described in "Materials and Methods." A, A representative autoradiogram is shown. B, Each histogram represents the average fold of 42-kD MAP kinase activities (the activity of control 42-kD MAP kinase is 1) from four independent experiments (mean \pm SE). * $P < .05$ vs stretch without any pretreatment.

after stretching of cardiomyocytes. Maximum activation of Raf-1 was induced in response to mechanical stretch for 2 minutes, as reported previously.² Pretreatment with 10^{-5} mol/L HOE 694 suppressed stretch-induced activation of Raf-1 by $\approx 50\%$ (Fig 3). Next, to clarify the dependency of MAP kinases on Raf-1 in the stretched myocytes, a MEK1-specific inhibitor PD 98059 was used. Mechanical stretch-induced MAP kinase activation was completely blocked by pretreatment with PD 98059 (Fig 4), suggesting that mechanical stretch induces sequential activation of Raf-1, MEK1, and MAP kinases.

Partial Blockade of Stretch-Induced Increase in Phenylalanine Uptake by HOE 694

We previously reported that stretching of cardiomyocytes increases protein synthesis.^{27,29} To elucidate involvement of the NHE in stretch-induced cardiomyocyte hypertrophy, we examined the relative protein synthesis by measuring incorpo-

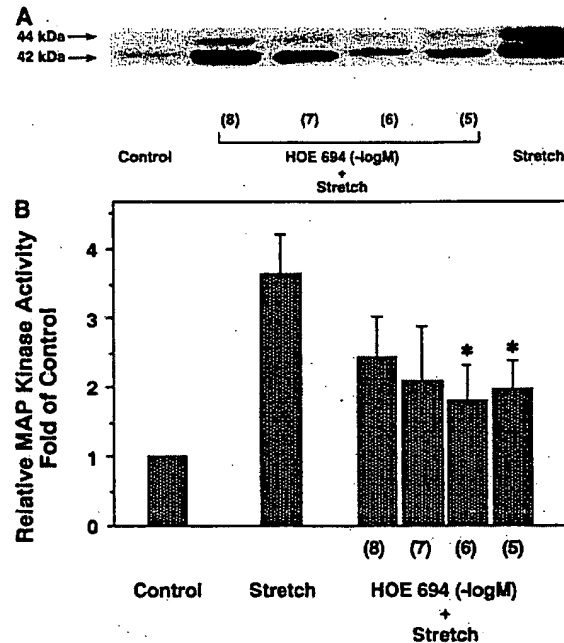


Figure 2. Dose-dependent inhibition of HOE 694 on stretch-induced MAP kinase activation. After pretreatment with 10^{-8} to 10^{-5} mol/L HOE 694 for 30 minutes, cardiomyocytes were stretched by 20% for 8 minutes. MAP kinase activities were measured as described in "Materials and Methods." A, A representative autoradiogram is shown. B, Each histogram represents the average fold of 42-kD MAP kinase activities (the activity of control 42-kD MAP kinase is 1) from four independent experiments (mean \pm SE). * $P < .05$ vs stretch without HOE 694 treatment.

ration of [3 H]phenylalanine into proteins. Stretch by 20% for 24 hours stimulated an increase in amino acid uptake by ≈ 1.5 -fold (Fig 5). Although pretreatment with HOE 694 did not affect basal [3 H]phenylalanine incorporation, stretch-induced increase in amino acid uptake was partially suppressed by the pretreatment with HOE 694. Other blockers such as gadolinium, streptomycin, glibenclamide, and CsCl did not have any effects on stretch-induced increase in amino acid uptake (data not shown). These results suggest that the development of stretch-induced cardiomyocyte hypertrophy partly depends on activation of the NHE and that HOE 694 has no nonspecific toxic effect on cardiac myocytes.

Cytoplasmic Alkalization Induced by Stretch Mediates MAP Kinase Activation

We have demonstrated that mechanical stress induces the activation of Raf-1 and MAP kinases maximally at 2 minutes and at 8 to 10 minutes after stretch, respectively.^{1,2,38} To ascertain whether full activation of Raf-1 at 2 minutes after stretch is enough to activate MAP kinases, cardiomyocytes were stretched for 2 minutes and kept unstretched for 6 minutes. Even under this condition, mechanical stretch activated MAP kinases, but the activities were lower than those induced by stretch for 2 minutes (Fig 6). Although the precise mechanisms remain uncertain, these results suggest that the continuous stretch is necessary for full activation of MAP kinases.

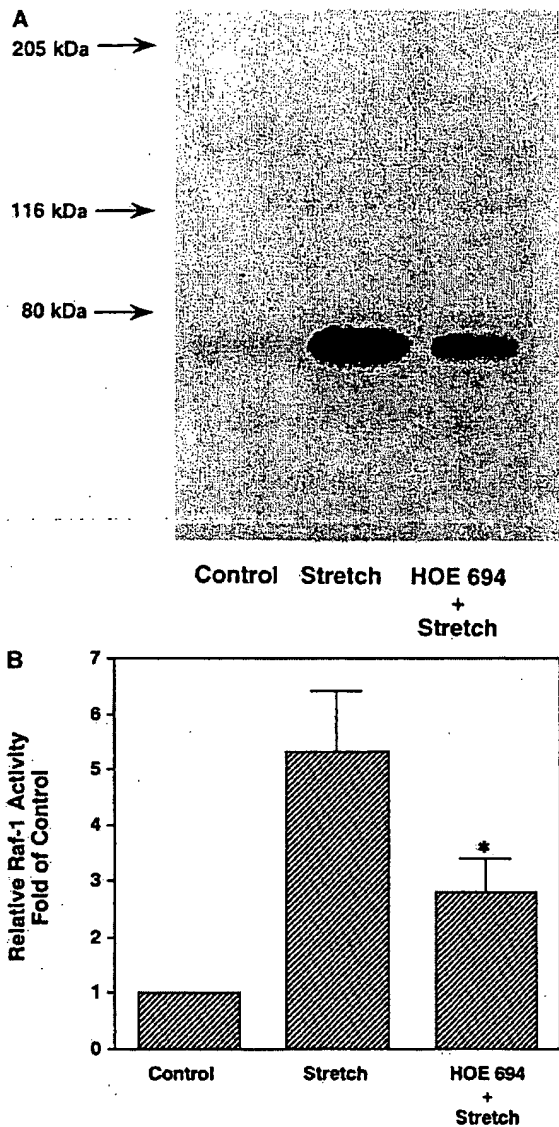


Figure 3. Effects of HOE 694 on stretch-induced Raf-1 activation. After pretreatment with 10^{-5} mol/L HOE 694 for 30 minutes, cardiomyocytes were stretched by 20% for 2 minutes. Raf-1 activities were measured by using rMAPKK as a substrate as described in "Materials and Methods." A, A representative autoradiogram is shown. B, Each histogram represents the average fold of Raf-1 activities (the activity of control Raf-1 is 1) from four independent experiments (mean \pm SE). * $P < .05$ vs stretch without HOE 694 treatment.

To further elucidate whether cytoplasmic alkalization evoked by the activated NHE mediates mechanical stretch-induced hypertrophic responses, MAP kinase activities were analyzed in acid-loaded cardiomyocytes.³⁹ Stretch-induced MAP kinase activation was significantly but partially inhibited by pretreatment with 4×10^{-2} mol/L NH_4Cl (Fig 6), suggesting that the cytoplasmic alkalization possibly induced by activation of the NHE may be a critical step to activate MAP kinases in the stretched myocytes.

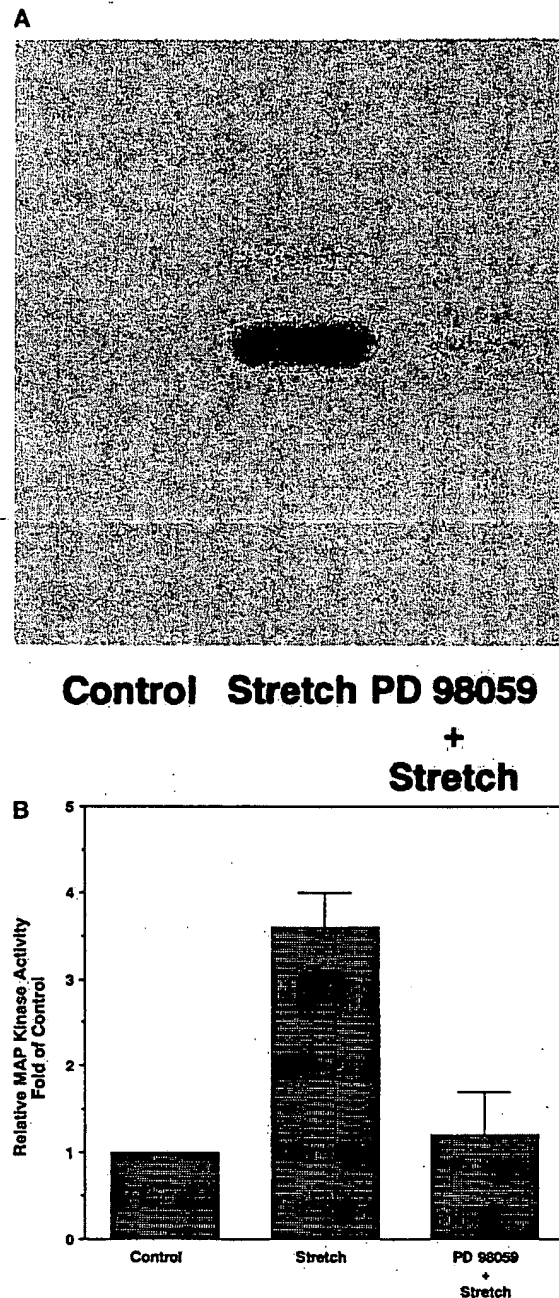


Figure 4. Effects of PD 98059 on stretch-induced MAP kinase activation. After pretreatment with 5×10^{-5} mol/L PD 98059 for 30 minutes, cardiomyocytes were stretched by 20% for 8 minutes, and MAP kinase activities were measured. A, A representative autoradiogram is shown. B, Each histogram represents the average fold of 42-kD MAP kinase activities (the activity of control 42-kD MAP kinase is 1) from four independent experiments (mean \pm SE).

Relationship Between the NHE and Autocrine Factors

We and others have recently reported that mechanical stretch stimulates secretion and production of Ang II and ET-1 in cardiomyocytes and that both factors are related to the devel-

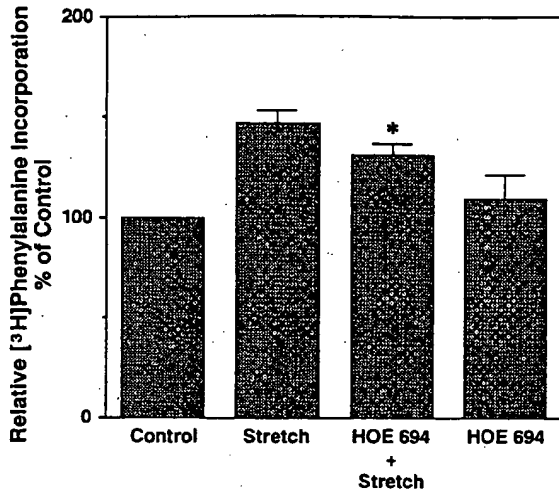


Figure 5. Effects of HOE 694 on stretch-induced increase in phenylalanine incorporation. Thirty minutes after pretreatment with HOE 694 (10^{-5} mol/L), cardiomyocytes were stretched by 20% for 24 hours. [3 H]Phenylalanine was added 2 hours before harvest, and the total radioactivity incorporated into proteins was determined by liquid scintillation counting. Each histogram represents the average percentage of control (=100%, no stimulus) from four independent experiments. * $P < .05$ vs stretch.

opment of myocardial hypertrophy induced by mechanical stretch.^{3,5,6} Thus, we analyzed the relationship between the NHE and these vasoactive peptides in the stretched myocytes. Consistent with the previous report,⁶ pretreatment with both the endothelin type A receptor antagonist BQ 123 and the Ang II type 1 receptor antagonist CV 11974 significantly inhibited mechanical stretch-induced MAP kinase activation (Fig 7). Moreover, addition of HOE 694 almost completely blocked the activation (Fig 7). These results suggest that there are at

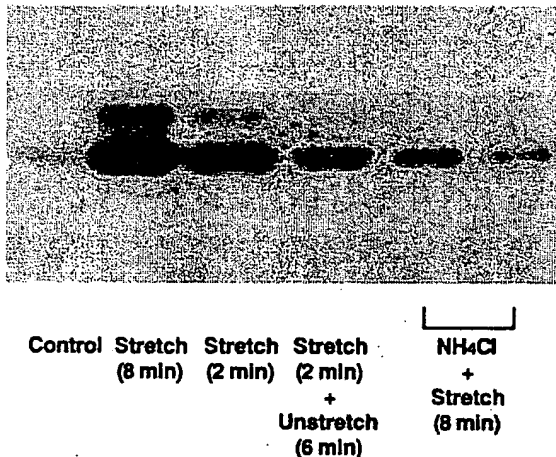


Figure 6. Continuous stretch and cytoplasmic alkalization-dependent MAP kinase activation. Cardiac myocytes were stretched by 20% for 2 minutes and then were kept unstretched for 6 minutes. In addition, cardiomyocytes were stretched by 20% for 8 minutes in the medium containing 4×10^{-2} mol/L NH_4Cl . MAP kinase activities were measured as described in "Materials and Methods." Similar results were obtained from three independent experiments, and a representative autoradiogram is shown.

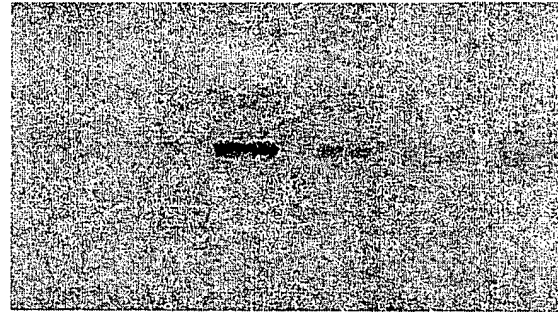


Figure 7. Effects of BQ 123, CV 11974, or HOE 694 on stretch-induced MAP kinase activation. After pretreatment with BQ 123 (10^{-5} mol/L), CV 11974 (10^{-6} mol/L), and HOE 694 (10^{-5} mol/L) for 30 minutes, cardiomyocytes were stretched by 20% for 8 minutes, and MAP kinase activities were measured. A representative autoradiogram from three independent experiments is shown.

least two distinct signaling pathways leading to MAP kinase activation in cardiac myocytes: the autocrinely released peptide-dependent pathway and the activated NHE-dependent pathway.

Furthermore, to ascertain whether these vasoactive peptides are involved in activation of the NHE in response to stretch, we examined the effect of HOE 694 on Raf-1 and MAP kinase activation induced by Ang II or ET-1. Ang II and ET-1 strongly increased the activity of Raf-1 by 2.4- and 3.0-fold, respectively (Fig 8A). Neither pretreatment with HOE 694 nor acid load inhibited Ang II- or ET-1-induced Raf-1 activation (Fig 8A). Nor was MAP kinase activation by Ang II or ET-1 attenuated by pretreatment with HOE 694 (data not shown) or NH_4Cl (Fig 8B). These results suggest that the NHE activated by mechanical stretch induces hypertrophic responses independently of autocrinely released Ang II and ET-1.

Discussion

Membrane proteins such as ion channels and exchangers have been reported to respond first to external stimuli.⁷ Mechanosensitive ion channels are especially good candidates for mechanoreceptors, which receive mechanical stress and evoke hypertrophy-inducing signals in cardiac myocytes. In the present study, however, stretch-induced biochemical events, such as activation of protein kinases and an increase in protein synthesis, were not inhibited by any blockers of mechanosensitive cation channels. In contrast, the NHE inhibitor HOE 694 drastically inhibited stretch-induced hypertrophic responses, including sequential activation of Raf-1 and MAP kinases followed by increased protein synthesis. Since activation of Raf-1 and MAP kinases by Ang II and ET-1 was not inhibited by HOE 694, the mechanical stress-induced activation of the NHE is likely to be independent of autocrinely secreted Ang II or ET-1.

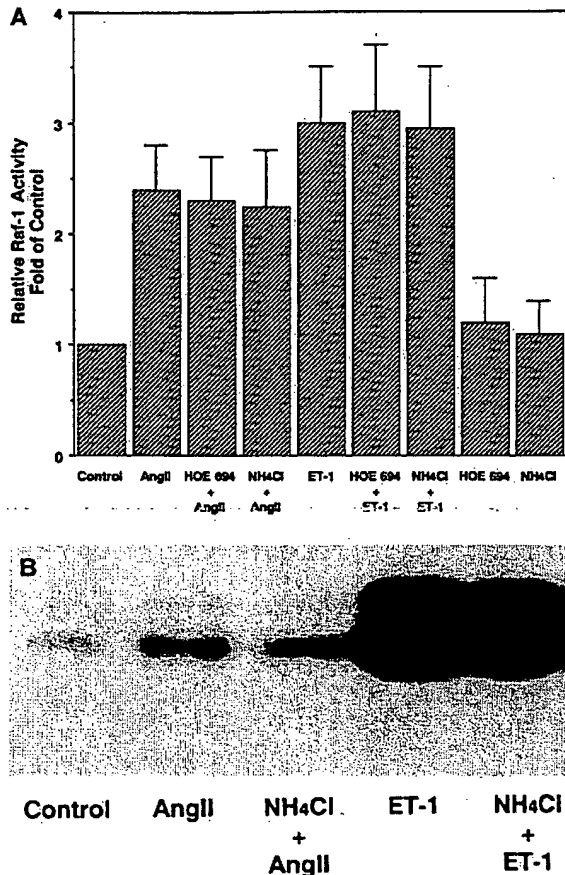


Figure 8. Effects of HOE 694 or extracellular acid load on Ang II- or ET-1-induced Raf-1 and MAP kinase activation. After pretreatment with 10^{-5} mol/L HOE 694 for 30 minutes or in the medium containing 4×10^{-2} mol/L NH_4Cl , cardiomyocytes were stimulated with Ang II (10^{-7} mol/L) or ET-1 (10^{-7} mol/L) for 2 minutes (A) or 8 minutes (B), respectively. Raf-1 activities (A) or MAP kinase activities (B) were measured by using syntide-2 (A) or MBP (B) as a substrate, respectively. A, Each histogram represents the average fold of control ($=100\%$) from four independent experiments (mean \pm SE). B, A representative autoradiogram from three independent experiments is shown.

Guharay and Sachs⁴⁰ first characterized mechanosensitive ion channels in the chick skeletal muscle; these channels are ubiquitously distributed in all organs, including cardiomyocytes, and have been reported to play critical roles in fundamental cell functions.⁷ By using many kinds of specific inhibitors, the roles of these ion channels in cardiac myocytes have been investigated.¹¹⁻¹⁵ It has been reported that gadolinium, a blocker of nonselective cation channels, does not affect either the stretch-induced expression of immediate-early response genes or the increase in protein synthesis.³⁴ In the present study, we have demonstrated that not only gadolinium but also glibenclamide, a blocker of ATP-sensitive K^+ channels, and CsCl, a blocker of hyperpolarization-activated inward channels, do not affect mechanical stretch-induced hypertrophic responses, ie, activation of Raf-1 and MAP kinases and an increase in protein synthesis.

The activity of the NHE is tightly regulated by its phosphorylation and dephosphorylation.⁴¹ A variety of mitogens

such as phorbol myristate acetate, epidermal growth factor, thrombin, and serum activate the NHE by phosphorylation.^{17,18} Recent reports have shown that 90-kD S6 kinase has strong NHE phosphorylation activity and that the kinase activity in vascular smooth muscle cells is higher in spontaneously hypertensive rats than in Wistar-Kyoto normotensive rats.^{42,43} On the other hand, cAMP inhibits the NHE-3 in the presence of a regulatory protein, E3KARP or NHERF.⁴⁴ The present results suggest that mechanical stretch activates the NHE in cardiac myocytes. It is of interest to determine how mechanical stretch activates the NHE. FAK and paxillin are tyrosine-phosphorylated in response to cell adhesion to and spreading on an extracellular matrix substrate.⁴⁵⁻⁴⁸ We have recently reported that by using the same system as in the present study, stretching of mesangial cells phosphorylates FAK.⁴⁹ Bockholt and Burridge⁵⁰ have also reported that cell spreading induces tyrosine phosphorylation of tensin, a focal adhesion protein, in rat embryonic fibroblasts. Since FAK is a tyrosine kinase concentrated in focal adhesion^{48,51} and tensin contains an SH2 domain that binds to phosphorylated tyrosine residues,⁵² sequential activation of these focal proteins may induce the protein kinase cascade that leads to the NHE activation. Actually, it has been reported that cell spreading induces activation of the NHE in 3T3 cells.¹⁹ Furthermore, a close interaction of fibronectin and the integrin receptor $\alpha 5 \beta 1$ has been shown to activate the NHE.⁵³ In addition to tyrosine kinase pathways, activation of G proteins by injecting a GTP analogue also activates the NHE of unfertilized sea urchin eggs⁵⁴ or *Xenopus laevis* oocytes.⁵⁵ Therefore, it remains to be determined which kinase(s) activates the NHE during mechanical stress in cardiac myocytes.

Which downstream signaling pathway can be exerted by the activated NHE is another important question. Integrin-mediated cell spreading, which activates the NHE,⁵³ causes phosphorylation and activation of MAP kinases in 3T3 fibroblasts.⁵⁶ We have demonstrated in the present study that activation of the Raf-1/MAP kinase pathway induced by stretch partly depends on the NHE. Activation of the NHE increases H^+ efflux and Na^+ influx, which may result in an increase in Ca^{2+} influx by the $\text{Na}^+-\text{Ca}^{2+}$ exchange mechanism. Many laboratories, including ours,^{57,58} have shown that Ca^{2+} plays an important role in activation of MAP kinases in cardiac and smooth muscle cells. Therefore, the NHE may be involved in the stretch-induced activation of Raf-1 and MAP kinases through increasing Ca^{2+} .

The NHE regulates cytoplasmic pH, which is one of the most important aspects of cellular homeostasis in all eukaryotic cells.¹⁶ Activation of the NHE leads to cytoplasmic alkalization, which persists as long as the activating signals exist,⁵⁹ and the increase in cytoplasmic pH by ≈ 0.15 is sufficient to increase the rate of protein synthesis by $\approx 35\%$ to 40% .⁶⁰ It has also been shown that an increase in cytoplasmic pH is responsible for the stimulation of protein synthesis in both perfused rat hearts⁶¹ and quiescent isolated cardiomyocytes.⁶² Although the mechanisms by which the cytoplasmic alkalization increases protein synthesis rates remain unknown, activation of the NHE in the stretched myocytes may induce myocardial hypertrophy by increasing cytoplasmic pH. This idea is supported by the experiments showing that extracellular

acidification strongly inhibited stretch-induced MAP kinase activation (Fig 6). On the other hand, pretreatment with HOE 694 did not affect basal phenylalanine incorporation by itself (Fig 5), suggesting that HOE 694 has no nonspecific toxic effect and that the NHE is inactivated in the unstretched myocytes.

Ang II stimulates an amiloride-sensitive NHE system in cultured vascular smooth muscle cells.⁶³ Endothelin also increases Na^+ uptake via the NHE in endothelial cells of brain microvessels.⁶⁴ Since secretion of Ang II and ET-1 is induced by stretch in cultured cardiac myocytes,^{3,5,6} we first hypothesized that Ang II and ET-1, of which secretion is induced by stretch, can activate the NHE by an autocrine mechanism. In the present study, however, Ang II- or ET-1-induced hypertrophic responses, such as activation of Raf-1 and MAP kinases, were not affected by HOE 694. In this regard, Berk et al⁶⁵ have clarified differences of the NHE activity in hyperplastic and hypertrophied vascular smooth muscle cells. Hyperplastic stimuli such as platelet-derived growth factor and serum induce the persistent activation of the NHE, whereas hypertrophic stimuli such as Ang II have no effect on the NHE activity and cause a decrease in cytoplasmic pH. Rao et al⁶⁶ have also shown that expression levels of the NHE mRNA in vascular smooth muscle cells are different in the growth state; ie, hyperplastic agonists increase steady-state levels of the NHE mRNA, whereas a hypertrophic agonist, such as Ang II, does not. Moreover, Fliegel et al⁶⁷ have shown that the cardiac NHE lacks consensus sequences for phosphorylation by PKC, a major second messenger of Ang II in cardiac myocytes.⁵⁸ Although there is no direct evidence at present, these observations and the present results suggest that Ang II or ET-1 may not activate the NHE in cardiac myocytes. Further studies are necessary to elucidate the cytoplasmic signaling pathways that exist upstream and downstream of the NHE in response to mechanical stress.

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